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Derivatives of activated H-*ras* lacking C-terminal lipid modifications retain transforming ability if targeted to the correct subcellular location

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To examine the ability of *ras* to activate signal transduction pathways in the absence of lipid modifications, fusion proteins were constructed that target ras^{WT} or activated ras^{61L} to cellular membranes as integral membrane proteins, using the first transmembrane domain of the E1 protein of avian infectious bronchitis virus (IBV), which contains a *cis*-Golgi targeting signal. Golgi-targeted derivatives of activated *ras* were completely inactive in transformation assays. However, when examined in focus formation assays, transformation of NIH3T3 cells were seen with derivatives of ras^{61L} containing a mutated E1 targeting sequence that results in plasma membrane localization. Removal of the lipid modification sites in and upstream of the CAAX motif did not abrogate the transforming activity of plasma membrane-localized ras^{61L} derivatives, indicating that these lipid modifications are not essential for *ras* activity, as long as the protein is correctly localized to the plasma membrane. Interestingly, the activity of integral membrane versions of ras^{61L} was strictly dependent on a minimum distance between the transmembrane domain anchor region and the coding sequence of *ras*. Derivatives with only a 3-amino acid linker were inactive, while linkers of either 11- or 22-amino acids were sufficient to restore transforming activity. These results demonstrate that: (1) activated *ras* targeted to Golgi membranes is unable to cause transformation; (2) lipid modifications at the C-terminus are not required for the transforming activity of plasma membrane-anchored ras^{61L} derivatives, and serve primarily a targeting function; (3) a transmembrane domain can effectively substitute for C-terminal modifications that would normally target *ras* to the inner surface of the plasma membrane, indicating that ras^{61L} does not need to reversibly dissociate from the membrane as might be allowed by the normal lipidation; and (4) in order to function properly, there exists a critical distance that the *ras* protein must reside from the plasma membrane.

Keywords: *ras*; subcellular localization; transformation

Introduction

ras proteins are critical modulators of signaling pathways involved in cell growth and differentiation. p21*ras* is a member of a large family of small proteins that act as molecular 'switches', cycling between an active GTP-bound state and an inactive GDP-bound state (Bourne *et al*., 1990). *ras* is activated in response to a wide variety of stimuli, including platelet-derived growth factor (PDGF). Binding of PDGF to its receptors induces their dimerization and transphosphorylation, creating binding sites for effector molecules (reviewed in Hart *et al*., 1995), some of which regulate or activate *ras*. Activation of *ras* by growth factors recruits *Raf*-1 to the plasma membrane (Stokoe *et al*., 1994; Leevers *et al*., 1994), leading to *Raf*-1 activation and subsequent initiation of the MAPK cascade. *ras* also has been shown to activate Rac and Rho proteins, which control signaling pathways that lead to cytoskeletal modifications (Ridley *et al*., 1992; Khosravi-Far *et al*., 1995; Qiu *et al*., 1995a,b). Activation of this pathway has recently been shown to be crucial for full transformation by *ras* (Prendergast *et al*., 1995; Khosravi-Far *et al*., 1995; Qiu *et al*., 1995a,b). Thus, combined activation of the Rac/Rho and MAPK pathways may constitute the entire intracellular response necessary for transformation by activated *ras*.

There are multiple p21 *ras* proteins, including N-*ras*, K-*ras*, and H-*ras* (reviewed in Santos and Nebreda, 1989; Lowy and Willumsen, 1993), which are localized to the inner surface of the plasma membrane (Willingham *et al*., 1980; Sefton *et al*., 1982) through specific sequences in the C-terminus (Willumsen *et al*., 1984). The mechanism by which *ras* proteins are targeted to the plasma membrane has been the subject of intense research. Since *ras* is frequently mutated in tumors, interference with *ras* localization could become an important means of treating some forms of cancer.

Post-translational modifications of *ras* proteins occur at the C-terminus, which includes a domain known as the 'CAAX' box, where A is an aliphatic amino acid, and X represents any amino acid. Processing of this region consists of farnesylation of the conserved Cys residue four amino acids from the C-terminus (Cys186 in H-*ras* and N-*ras*), cleavage of the last three amino acids of the protein, and carboxymethylation of the new C-terminal Cys (Hancock *et al*., 1989; Jackson *et al*., 1990; Casey *et al*., 1989; Gutierrez *et al*., 1989; Clarke *et al*., 1988; Lowy and Willumsen, 1986; Shih and Weeks, 1984; Willumsen *et al*., 1984; Sefton *et al*., 1982). N-*ras* and H-*ras* have cysteines N-terminal of the CAAX motif, which become palmitoylated after the CAAX processing (Buss and Sefton, 1986). In the case of K-*ras*-4B, the C-terminus contains a polybasic domain in addition to the CAAX motif which contributes to plasma membrane localization (Hancock *et al*., 1990, 1991; Jackson *et al*., 1994). The palmitoylation reactions are reversible, allowing for potentially dynamic localization of *ras* proteins (Magee *et al*.,

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1987). Although truncation of the C-terminus does not affect the inherent structure or biochemical properties of *ras* (John *et al*., 1989), mutations in these motifs render *ras* proteins cytosolic and inactive, implying that lipid modifications are important for *ras* function.

There is recent evidence suggesting that the lipid modifications of *ras* are also necessary for activation of *Raf*-1 and B-*Raf* (Okada *et al*., 1996; Kikuchi and Williams, 1994). However, since these lipid modifications are also responsible for targeting *ras* to the plasma membrane, it is unclear from these recent reports whether the lipid modifications *per se* are a necessity, or just localization of *ras* to the plasma membrane. The palmitoylation reactions that occur in the C-terminus of *ras* proteins have been shown to be reversible (Magee *et al*., 1987). There is also some speculation that *ras* may require reversible association with membranes, and the lipid modifications in the C-terminus provide the potential for this dynamic localization. However, the experimental methods used to date are unable to conclusively distinguish the precise role of lipid modifications in the membrane anchoring and activation of *ras*.

In order to examine these questions, we have used a novel method of targeting *ras* to the plasma membrane by creating fusion proteins using the transmembrane targeting signal from the E1 protein of avian IBV placed at the N-terminus (Swift and Machamer, 1991). In its wild-type form, this transmembrane domain is capable of targeting heterologous proteins to *cis*-Golgi membranes. When the point mutation Gln37→Ile is present, proteins are instead targeted to the plasma membrane. These fusions allow us to address the question of whether *ras* must be able to reversibly associate with the plasma membrane for biological function as assayed by transformation and GTPase activity. To examine whether C-terminal modifications of *ras* are required for functions other than targeting, we created similar fusion proteins that contain mutations which abolish processing of the CAAX box. Results presented here demonstrate that ras^{61L} can transform fibroblasts if targeted to the plasma membrane by an N-terminal transmembrane domain, yet is not active when localized to Golgi membranes. We also show that integral membrane versions of ras^{61L} require a minimum distance from the membrane in order to activate signal transduction pathways leading to transformation.

This work describes the first derivatives of ras^{61L} that lack any lipid modifications, and demonstrate that C-terminal lipid modifications are not required for transformation by ras^{61L}.

Results

Description of transmembrane-anchored ras *derivatives*

To examine the possibility that activated H-*ras* can transform if targeted to membranes by an N-terminal transmembrane anchor, a variety of fusion proteins were designed. As illustrated in the left side of Figure 1, ras^{WT} and ras^{61L} proteins are localized at the inner surface of the plasma membrane by means of lipid modifications at the C-terminus. The right side of Figure 1 depicts the orientation and design of the transmembrane-anchored *ras* derivatives. For derivatives containing the E1 *cis*-Golgi targeting signal, the transmembrane domain anchors the fusion proteins at Golgi membranes as Type I integral membrane proteins, with the *ras*-derived portion of the protein still in the cytosol. These derivatives allowed us to examine whether *ras* could function if localized to any membranous environment, thereby assessing the importance of the interaction between *ras* and the plasma membrane. Fusion proteins were also designed with a mutated E1(QI) targeting signal and are expected to be transported to the plasma membrane, with the N-terminus outside the cell, and the *ras* portion of the proteins on the inner surface of the plasma membrane.

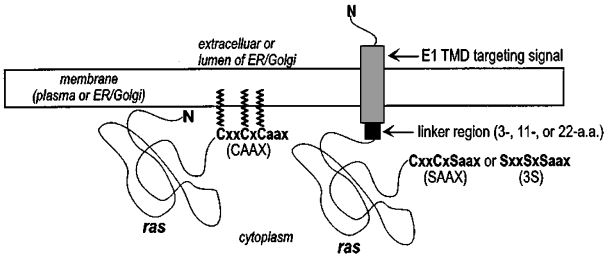

Figure 1 Schematic of *ras* fusion proteins. All fusion proteins have either the normal C-terminus (amino acids 181–189) of ras^{WT} or ras^{61L} (CMSCKCVLS), or contain SAAX (CMSCKSVLS) or 3S (SMSSKSVLS) mutations, which alter or abolish the normal post-translational processing and lipid modifications of the proteins. The N-terminus of each E1 derivative is oriented either into the lumen of the Golgi, or is extracellular if the point mutation Q→I is present. The whole of the *ras* protein is always cytosolic. The transmembrane domain (designated by the shaded box) is either the *cis*-Golgi targeting signal contained in the first transmembrane domain of the IBV E1 protein, or a mutated version (Q37→I) that targets the protein to the plasma membrane. The linker region between the transmembrane domain and the *ras* protein denoted by the black box is either 3-, 11-, or 22-amino acids in length

Figure 2 represents all of the fusion proteins designed for this study. The feasibility of targeting *ras* to the plasma membrane by the N-terminus was previously demonstrated in work by Buss *et al*. (1989) and Lacal *et al*. (1988), which examined the effect of N-terminal myristylation on ras^{61L} activity. Buss *et al*. (1989) described a naturally-occurring myristylated derivative of *ras*, p29Gag-*ras*, containing a 59-amino linker region between the myristylated Gly and the *ras* coding sequence. Lacal and colleagues generated N-terminally anchored *ras* derivatives by attaching the 15-amino acid myristylation signal from pp60c-*src*. Our derivatives differ in that *ras* is anchored permanently to membranes as an integral membrane protein. The myristylation targeting signal used in previous studies has the potential to allow reversible association of *ras* with the plasma membrane. This is an important distinction, which allows us to examine whether *ras* needs to be able to come off of the membrane in order to function. By exploiting the E1 localization signal, we were also able to target *ras* specifically to different membranes in the cell. We were also able to examine in more detail the minimum distance from the membrane that *ras* requires for activity, by designing constructs with linker regions of either 3, 11 or 22 amino acids.

Additional fusion proteins were designed to examine the general requirement for lipid modifications for *ras* activity. *ras* derivatives with 3- or 11-amino acid linkers

were constructed in the context of the unmutated (CAAX) C-terminus, as well as the SAAX and 3S C-terminal mutations. 'CAAX' refers to the wild-type C-terminal sequences of *ras*. 'SAAX' indicates a mutation of Cys186 in the CAAX motif to Ser, which abolishes the farnesylation, cleavage, and carboxymethylation reactions. '3S' refers to mutation of Cys181, 184, and 186 to Ser, which in addition destroys the palmitoylation sites (Hancock *et al*., 1989). The 22-amino acid linker derivatives were only made in the context of the SAAX mutant C-terminus. Since mutation of all three C-terminal Cys residues results in a *ras* protein completely unable to be modified by lipid moieties, these mutants allowed us to assay whether these lipid modifications are necessary for activity, or just utilized in a membrane targeting capacity.

ras *derivatives are unable to cause transformation if targeted to Golgi membranes*

When tested in focus forming assays, Golgi-targeted derivatives of ras^{WT} with 3-, 11-, or 22-amino acid

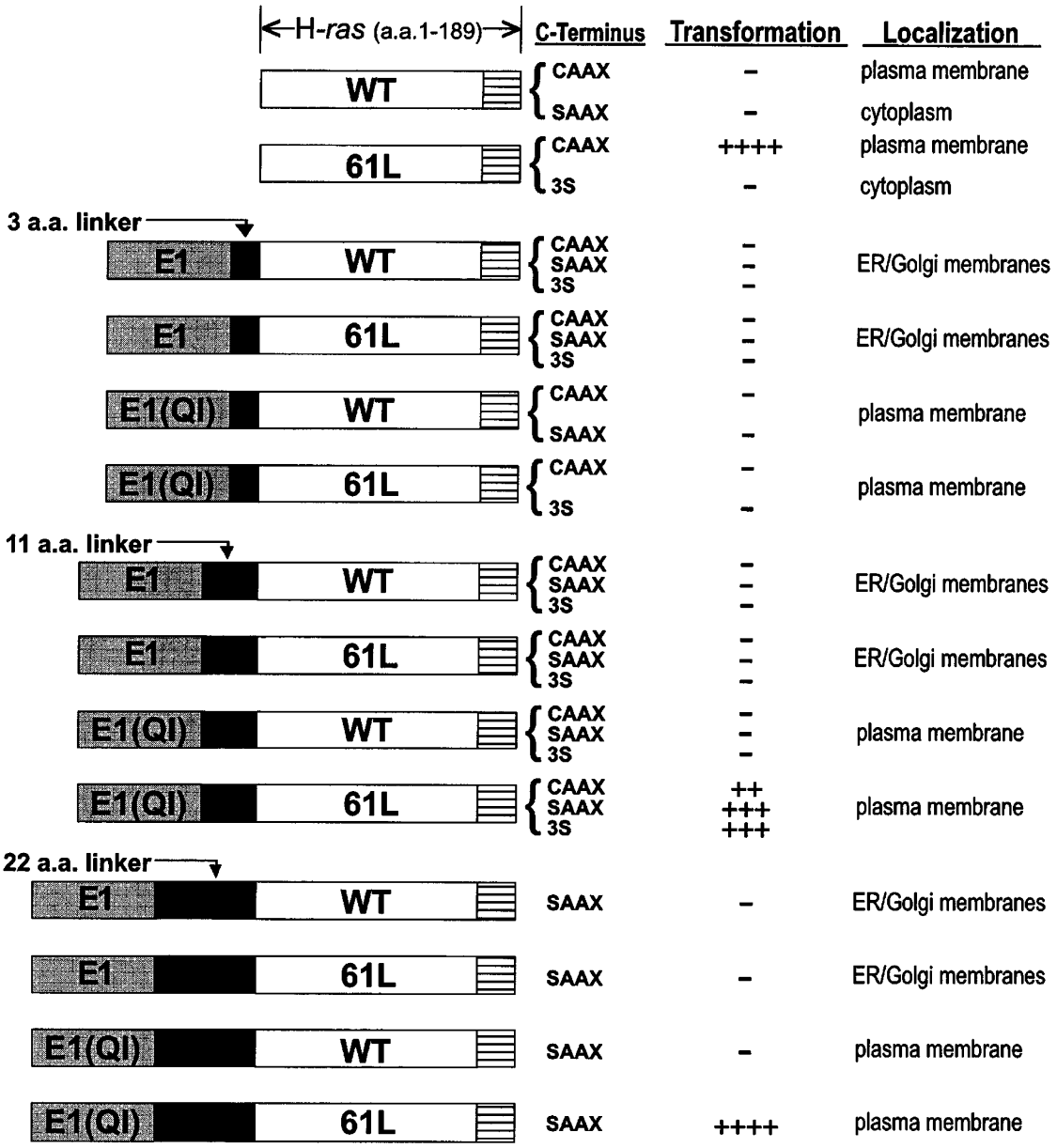

Figure 2 Localization and transforming activity of *ras* derivatives. Bars depict the ras^{WT} and ras^{61L} constructs tested in this paper. The following abbreviations are used to describe the different derivatives. '61L' refers to mutation of codon 61 of *ras* from Gln to Leu, which oncogenically activates the protein (Sekiya *et al*., 1984). 'E1' refers to the first transmembrane domain (amino acids 1–45) of the E1 protein of avian IBV, which constitutes a *cis*-Golgi targeting signal that can target heterologous proteins to the Golgi (Swift and Machamer, 1991). 'E1(QI)' indicates a point mutation in the transmembrane domain (Gln37→Ile) that abrogates its Golgi-targeting function, targeting proteins instead to the plasma membrane (Swift and Machamer, 1991). Shaded boxes indicate the E1 or E1(QI) transmembrane domain; black boxes indicate the linker region; striped boxes indicate the C-termini of the clones which is either wild-type (CAAX) or mutant (SAAX or 3S). Localization was determined by indirect immunofluorescence, as described in the legend for Figure 4. Transforming activity of the clones was analyzed in a focus forming assay, where NIH3T3 cells were transfected with the plasmids encoding the fusion proteins, and examined for foci after approximately 12–14 days. Each experiment was repeated at least three times for transforming derivatives, and at least two times for non-transforming constructs. Results are expressed as an average of all experiments performed with each given construct. $-$ = 0–5% of ras^{61L}; $++$ = 20–25% of ras^{61L}; $+++$ = 50–60% of ras^{61L}; $++++$ = 80–100% of ras^{61L} transforming activity

linker regions were all inactive. This was expected, since *ras*^{WT} did not induce focus formation in our assays. The Golgi-targeted derivatives of *ras*^{61L} were also unable to cause transformation. This was irrespective of whether the linker region was 3-, 11-, or 22-amino acids in length, or whether the C-terminal post-translational modification signals were intact or not.

Figure 4E and I demonstrate localization of E1-11-*ras*^{61L}-SAAX and E1-22-*ras*^{61L}-SAAX, respectively, to a perinuclear region typical of Golgi staining. Panel K shows the localization of E1-11-*ras*^{61L}-CAAX, and Figure 4L shows that this fusion protein colocalizes with mAb 10E6 (see arrows), which specifically reacts with a *cis*-Golgi epitope, and has been used as a marker for the early Golgi (Hart *et al*., 1994; Wood *et al*., 1991). This confirms that the Golgi-targeted fusion proteins are localized correctly, and therefore we conclude that *ras*^{61L} cannot initiate transforming signal transduction pathways from Golgi membranes, indicating that attachment to any lipid-filled environment is not sufficient for *ras* to be active.

ras^{61L} *derivatives with 11- or 22-amino acid linkers can transform if anchored to the plasma membrane, irrespective of C-terminal lipid modifications*

The plasma membrane-targeted derivatives of *ras*^{61L} with the 3-amino acid linkers (E1(QI)-3-*ras*^{61L}-CAAX, E1(QI)-3-*ras*^{61L}-SAAX, and E1(QI)-3-*ras*^{61L}-3S) were all inactive in focus formation assays. However, derivatives with 11- or 22-amino acid linker regions were very active in transformation of NIH3T3 cells. The results of these assays are summarized in Figure 2, which indicates the focus forming activity of each *ras* derivative as an average of at least three independent experiments. E1(QI)-11-*ras*^{61L}-CAAX, E1(QI)-11-*ras*^{61L}-SAAX, E1(QI)-11-*ras*^{61L}-3S and E1(QI)-22-*ras*^{61L}-

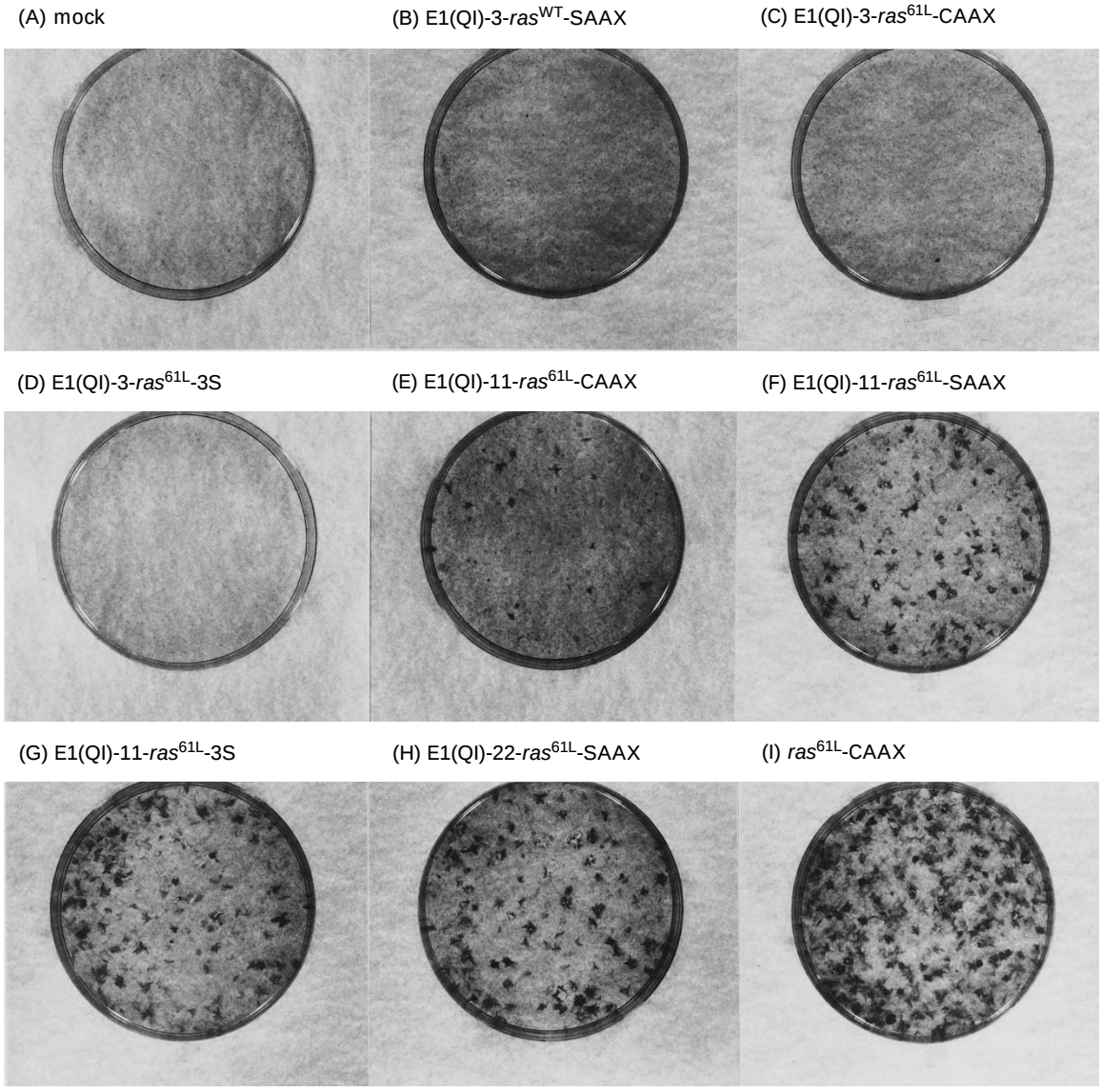

Figure 3 Focus forming assays of *ras* derivatives targeted to the plasma membrane as integral membrane proteins. Plasmids encoding *ras* fusion proteins were transfected into NIH3T3 cells, and foci were counted after 12–14 days. Cells were fixed with methanol, stained with Giemsa, and photographed. (**A**) mock-transfected cells; (**B**) E1(QI)-3-*ras*^{WT}-SAAX; (**C**) E1(QI)-3-*ras*^{61L}-CAAX; (**D**) E1(QI)-3-*ras*^{61L}-3S; (**E**) E1(QI)-11-*ras*^{61L}-CAAX; (**F**) E1(QI)-11-*ras*^{61L}-SAAX; (**G**) E1(QI)-11-*ras*^{61L}-3S; (**H**) E1(QI)-22-*ras*^{61L}-SAAX; (**I**) *ras*^{61L}-CAAX

SAAX exhibited significant transforming activity in comparison with ras^{61L} (See Figure 3E–I). It is of interest to note that mutations in the C-terminus of ras^{61L} that abolish the lipidation of the proteins do not affect the ability of activated *ras* to transform NIH3T3 cells. Indeed, the derivative with an intact C-terminus was consistently three- to four-fold less efficient at transformation than derivatives with mutated C-termini (See Figure 2; also compare Figure 3E, F and G). This could reflect conformational strains conferred on the protein due to anchoring both termini to the membrane.

Plasma membrane-targeted derivatives containing 3-, 11-, or 22-amino acid linkers were also examined by immunofluorescence. As seen in Figure 4, derivatives that were positive in transformation assays localize to the plasma membrane (F, G, H, and J), and exhibit staining patterns comparable to that of ras^{61L} (B). Also, derivatives with 3-amino acid linkers were still targeted to the plasma membrane (D), although unable to transform cells. Thus, derivatives of ras^{61L} targeted to the plasma membrane via an N-terminal transmembrane domain are able to cause transformation, provided that there is a flexible linker region of sufficient length between the transmembrane anchor and the N-terminus of *ras*. These results also clearly demonstrate that ras^{61L} does not require lipid modifications in order to transform cells, and suggests that the C-terminal processing of *ras* proteins primarily functions in a membrane-anchoring capacity.

Immunoprecipitation demonstrates correct expression of fusion proteins

In order to verify that the fusion proteins being expressed were of the correct size, proteins were immunoprecipitated from transiently transfected cells (Figure 5). *ras* proteins typically run as a doublet on SDS–PAGE gels, one band representing the unprocessed form of *ras*, the other indicating the mature, processed form of the protein (see arrows) (Shih *et al*., 1980, 1982). The SAAX and 3S mutations prevent the C-terminal cleavage step and subsequent lipid modifications from occurring, leaving only a single band (lanes 3 and 4, arrows).

The subtleties of these processing steps are difficult to detect in the remaining fusion proteins shown in Figure 5, due to their increased molecular weight. However, it is quite apparent that all fusion proteins do express the additional N-terminal transmembrane domain sequences (lanes 5–12), and these targeting domains are not cleaved off during maturation of the proteins. The fusion proteins migrate slower than

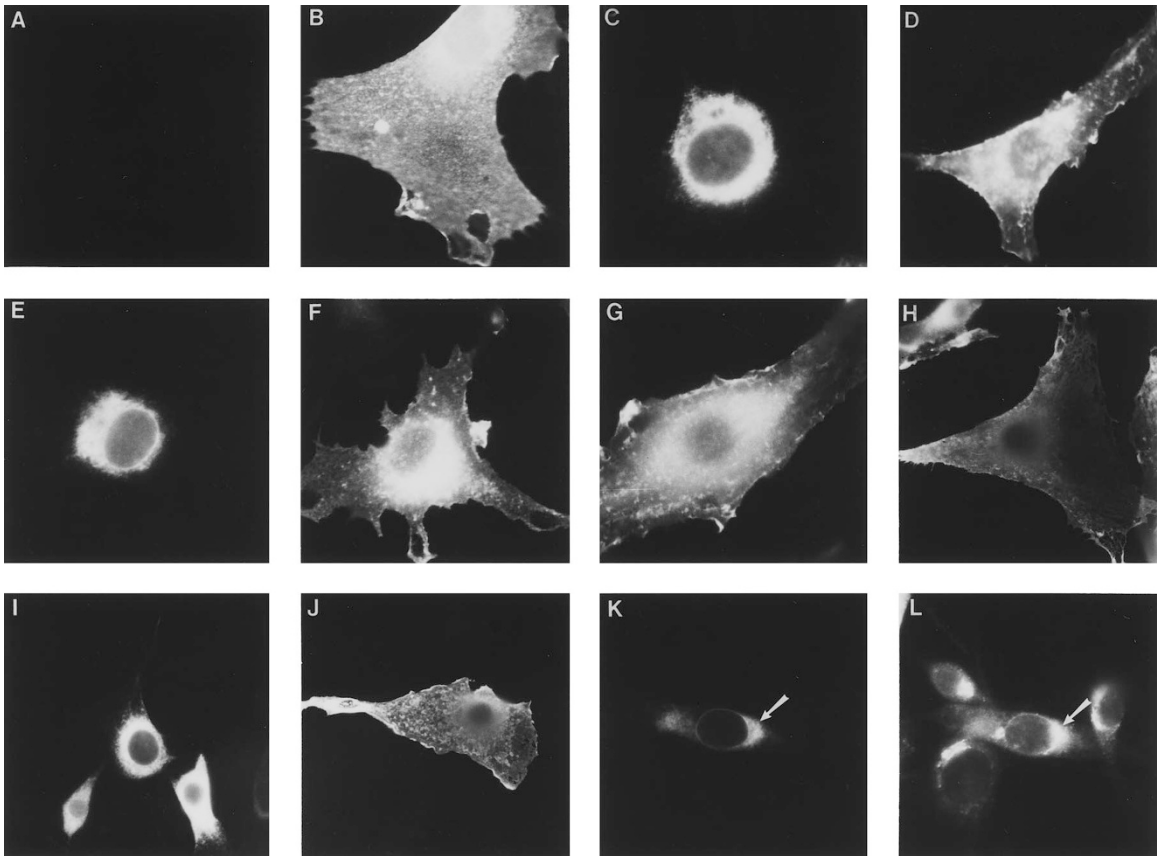

Figure 4 Indirect immunofluorescence confirms localization of *ras* derivatives. NIH3T3 cells were transiently transfected with various *ras* constructs, fixed, permeabilized, and incubated with rat mAb Ab-2 (clone Y13-238) and fluorescein-conjugated goat α-rat secondary antibody. For (**L**) mAb 10E6 was used to detect the *cis*-Golgi, and visualized with rhodamine-conjugated goat α-mouse secondary antibody. (**A**) mock; (**B**) ras^{61L}-CAAX; (**C**) E1-3-ras^{WT}-3S; (**D**) E1(QI)-3-ras^{61L}-3S; (**E**) E1-11-ras^{61L}-SAAX; (**F**) E1(QI)-11-ras^{61L}-CAAX; (**G**) E1(QI)-11-ras^{61L}-SAAX; (**H**) E1(QI)-11-ras^{61L}-3S; (**I**) E1-22-ras^{61L}-SAAX; (**J**) E1(QI)-22-ras^{61L}-SAAX; (**K**) E1-11-ras^{61L}-CAAX - α-ras antibody (fluorescein); (**L**) E1-11-ras^{61L}-CAAX - mAb 10E6 α-*cis*-Golgi antibody (rhodamine). Arrows indicate CAAX - mAb in **K** and **L**

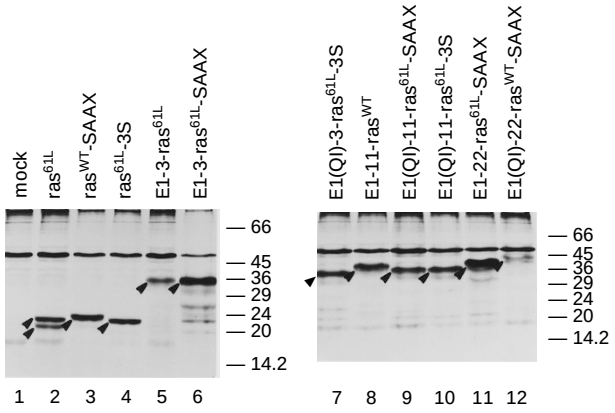

Figure 5 Immunoprecipitation of *ras* proteins demonstrates expression of fusion proteins and size differences corresponding to increasing linker length. NIH3T3 cells transiently transfected with various *ras* derivatives were labeled for 2 h with [^{35}S]-Cys and [^{35}S]-Met, lysed, and subjected to immunoprecipitation with mAb Ab-2 (Y13-238) against v-H-*ras*. Proteins were separated by 15% SDS–PAGE, and detected by autoradiography (exposure time = 2 days) Lane 1: mock; Lane 2: *ras*^{61L}-CAAX; Lane 3: *ras*^{WT}-SAAX; Lane 4: *ras*^{61L}-3S; Lane 5: E1-3-*ras*^{61L}-CAAX; Lane 6: E1-3-*ras*^{61L}-SAAX; Lane 7: E1(QI)-3-*ras*^{61L}-3S; Lane 8: E1-11-*ras*^{WT}-CAAX; Lane 9: E1(QI)-11-*ras*^{61L}-SAAX; Lane 10: E1(QI)-11-*ras*^{61L}-3S; Lane 11: E1-22-*ras*^{61L}-SAAX; Lane 12: E1(QI)-22-*ras*^{WT}-SAAX. Molecular weight standards in kD are indicated to the right of each gel. Arrows indicate position of fusion proteins

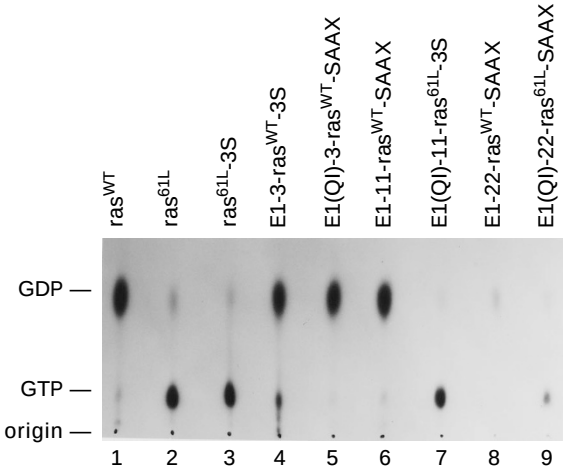

Figure 6 GTPase activity of transmembrane-anchored *ras* derivatives. Fusion proteins were immunoprecipitated with mAb Ab-2 (Y13-238) from transiently transfected NIH3T3 cells, and subjected to an *in vitro* GTPase assay with α-[^{32}P]GTP. Samples were spotted on PEI-Cellulose plates and chromatographed in 0.75 M KH_2PO_4, pH 3.4. Locations of GTP and GDP are indicated at the left of the figure. Note that all *ras*^{WT} derivatives have a higher GTPase activity (lanes 1, 4, 5, 6 and 8) compared to *ras*^{61L} derivatives (lanes 2, 3, 7 and 9), as evidenced by increased amount of labeled GDP versus GTP in *ras*^{WT} samples. This result is irrespective of localization to Golgi or plasma membranes, and regardless of C-terminal modifications. Lane 1: *ras*^{WT}-CAAX; Lane 2: *ras*^{61L}-CAAX; Lane 3: *ras*^{61L}-3S; Lane 4: E1-3-*ras*^{WT}-3S; Lane 5: E1(QI)-3-*ras*^{WT}-SAAX; Lane 6: E1-11-*ras*^{WT}-SAAX; Lane 7: E1(QI)-11-*ras*^{61L}-3S; Lane 8: E1-22-*ras*^{WT}-SAAX; Lane 9: E1(QI)-22-*ras*^{61L}-SAAX

expected, which may be due to the presence of an N-linked glycosylation site in the extreme N-terminus of the E1-derived sequence (Machamer *et al*., 1990).

GTPase activity of transmembrane-anchored ras *derivatives*

The transforming ability of some of the plasma membrane-targeted derivatives of *ras*^{61L} indicates that their function was not adversely affected by addition of an N-terminal transmembrane domain. To analyse whether fusion proteins that were non-transforming were still able to function in some manner, GTPase activity was examined. *ras*^{61L} remains locked in the GTP-bound state longer due to a decreased rate of GTP hydrolysis (Temeles *et al*., 1985), despite a 50-fold increased binding affinity for *ras*GAP (Krengel *et al*., 1990). If the N-terminal transmembrane domain does not interfere with normal protein function, one would expect to see a higher GTPase activity exhibited by *ras*^{WT} fusion proteins, as compared to *ras*^{61L} derivatives. Using thin-layer chromatography, this difference in GTPase activities is apparent, as shown by a higher proportion of labeled GDP versus GTP in *ras*^{WT} samples as compared to *ras*^{61L} samples (Figure 6, lanes 1 and 2).

Interestingly, *ras*^{61L} with the 3S mutation in the C-terminus, which renders the protein cytosolic and inactive in transformation, does not affect the GTPase activity (lane 3). Derivatives of *ras*^{WT} with the 3-amino acid linker hydrolyze GTP, irrespective of whether they are targeted to the plasma membrane (lane 5) or Golgi membranes (lane 4). *ras*^{61L} derivatives with transmembrane anchors and 3-amino acid linkers exhibit impaired GTPase activity as expected (data not shown). Finally, the 11- and 22-amino acid derivatives of *ras*^{WT} (lanes 6 and 8) or *ras*^{61L} (lanes 7 and 9) exhibit the expected GTPase activity profiles, regardless of the transmembrane domain attached at the N-terminus or the modifications present at the C-terminus. These results demonstrate that *ras* proteins can be targeted to the plasma membrane via a transmembrane anchor without affecting the expected GTPase activity of the proteins. Also, we find that C-terminal lipid modifications do not play a role in maintaining the intrinsic GTPase activity of wild-type or activated *ras*.

Discussion

C-terminal lipid modifications are not required for ras^{61L} *transforming activity*

The experiments described above demonstrate that *ras*^{61L} can activate transforming signal transduction pathways when targeted by the N-terminus to the plasma membrane as an integral membrane protein. This activity was completely independent of the normal post-translational processing that occurs at the C-terminus of *ras* proteins. Since *ras*^{61L} derivatives (E1(QI)-11-*ras*^{61L}-SAAX, E1(QI)-11-*ras*^{61L}-3S and E1(QI)-22-*ras*^{61L}-SAAX) lacking the C-terminal sequences required for normal processing of *ras* are still able to cause transformation, it is clear that lipid modifications *per se* are not required for activity of oncogenic *ras*. This is the first demonstration of non-lipidated transforming derivatives of *ras*.

Our data suggests that post-translational modification of H-*ras* serves primarily to target the protein to the plasma membrane. Whether there are other, more subtle functions of these C-terminal lipid modifications

distinct from their more obvious targeting function remains to be demonstrated. The observation that three of the transmembrane-anchored derivatives, E1(QI)-11-*ras*^{61L}-SAAX, E1(QI)-11-*ras*^{61L}-3S, and E1(QI)-22-*ras*^{61L}-SAAX, retain transforming activity despite their inability to be lipid-modified strongly implies that downstream signaling molecules such as *Raf*-1 and the MAPK cascade are activated in these cells. This contradicts recent reports that post-translational modifications of *ras* are required for activation of *Raf*-1 and B-*Raf* (Okada *et al*., 1996; Kikuchi and Williams, 1994). The precise activation state of effectors downstream from *ras*, and whether these molecules are activated in a similar fashion by integral membrane versions of *ras*, is currently under investigation.

Transforming activity of transmembrane derivatives of ras *demonstrates that* ras *does not require transient or reversible association with the membrane*

If the lipid modifications on *ras* proteins serve only a targeting function, as indicated by our results, this raises the question of why *ras* proteins evolved to contain C-terminal lipid modifications instead of a more permanent transmembrane anchor. Small GTP-binding proteins related to *ras* are now known to function in a variety of capacities within the cell, such as mediating vesicular transport in the process of endocytosis, sorting and trafficking through the secretory pathway, and regulating the structure of the cytoskeleton (reviewed in Hall, 1994; Ferro-Novick and Novick, 1993; Pfeffer, 1994; Zerial and Stenmark, 1993). Some of these processes may require the regulatory GTP-binding proteins to be transiently or reversibly associated with lipid bilayers, and only lipid modifications would allow this dynamic association to occur. Our results suggest, in the case of *ras*, that a reversible association with the plasma membrane is not required for activation of transforming signal transduction pathways, since integral membrane versions of *ras*^{61L} are transforming. It would be interesting to examine the effects of anchoring other small GTP-binding proteins more permanently to specific membranes or compartments using targeting sequences such as the transmembrane domain from the E1 protein.

ras *proteins require a minimum spacing from the plasma membrane in order to function*

Transforming activity of *ras*^{61L} derivatives was dependent upon a minimum distance from the plasma membrane mediated by the length of the linker region. Altering the distance between *ras* and the plasma membrane does affect the ability of *ras* to activate signal transduction pathways, as demonstrated by the inactivity of 3-amino acid linker derivatives of *ras*^{61L} and activation of these derivatives in transformation assays by addition of 11- or 22-amino acid linker regions. The crystal structure of c-H-*ras* bound to GDP (residues 1–171) or GTP (residues 1–166) indicates that the extreme C-terminus forms an alpha-helical region that juts out from the globular catalytic region of *ras* (De Vos *et al*., 1988; Pai *et al*., 1989), suggesting that the catalytic domain is well-removed from the membrane in normal *ras* proteins. One can imagine that the native C-terminus of *ras* constitutes a natural 'linker' region, not unlike the artificial linker regions incorporated into our *ras* fusion proteins. This would explain the failure of the short 3-amino acid linker at the N-terminus to allow for a functional fusion protein, whereas linkers of 11- or 22-amino acids, corresponding more closely in length to the C-terminus of normal *ras*, do result in a transforming *ras*^{61L} derivative.

Conformational energy analysis performed on the 18 C-terminal amino acids excluded from the crystal structure of the GDP-bound form of *ras* indicates that this region likely forms a helix–turn–helix or 'helical hairpin' motif, allowing the N-terminus and C-terminus of *ras* to be in close proximity (Brandt-Rauf *et al*., 1990). Perhaps these two regions of the protein interact in some manner. However, it appears from our results that interactions between the N-terminus and lipid modifications at the C-terminus are not important for *ras* function. There could be one or more effectors of *ras* that bind to a site different from the identified effector domain, and access to this site may be dependent upon orientation of the protein with respect to the membrane, or may require binding to sites at both the N- and C-terminus of *ras* proteins. Identification of other membrane-associated factors involved in regulation or activation of *ras* proteins and their effectors will provide clues as to why *ras* proteins evolved their unique structural elements.

GTPase activity of ras *derivatives*

Clearly the intrinsic GTPase activities of our fusion proteins are not altered when compared to normal versions of *ras*^{WT} and *ras*^{61L}. GTP binding and hydrolysis are also apparently unaffected by mutation of the CAAX box. Thus, it appears that neither the N- nor the C-terminus are important for this function of *ras*. Specific amino acids in the effector regions of *ras* (amino acids 32–38 or Switch I, and amino acids 60–76 or Switch II) are involved in association with guanine nucleotides and also in binding to regulators of GTPase and nucleotide exchange activities. Since GTPase activity is normal in the fusion proteins, the overall structure of the catalytic regions is not expected to be drastically altered. To our knowledge, this is the first study examining the role of C-terminal lipid modifications in regulating GTPase activity of *ras*.

One discrepancy between our results and those of Buss *et al*. (1989) is that anchoring of wild-type *ras* to the plasma membrane by the E1(QI) transmembrane domain does not result in transformation. Buss *et al*. (1989) found significant focus forming ability of wild-type *ras* after addition of N-terminal myristylation. They speculated that perhaps their protein was binding to inappropriate cellular targets, which may not be promoted by a transmembrane anchor. It is also possible that interaction with nucleotide exchange factors was enhanced in myristylated *ras*^{WT} derivatives, but this does not occur in E1(QI)-*ras*^{WT} constructs. To further clarify the role of membrane association in regulating the inherent enzymatic activity of *ras* proteins, it would be interesting to see if there

are any subtle effects on the association of integral membrane versions of *ras* proteins with regulators of GTPase and nucleotide exchange activities.

Implications for regulation and activation of ras

The observation that Golgi-targeted derivatives of *ras* are unable to transform fibroblasts, while perhaps not unexpected, provides some insight into the requirements for signal transduction through the *ras* pathway. It is clear that some mediators of the transforming pathway initiated by oncogenic *ras* are only available at the plasma membrane. Precisely what these mediators are remains to be elucidated. However, one can conclude that simple juxtaposition of *ras* to a lipid-rich environment is not sufficient to allow it to signal. Examination of these Golgi-targeted *ras* derivatives and their ability to interact with or activate the normal cytosolic substrates such as *Raf*-1 will provide future insights into the function of *ras*.

Recent evidence demonstrates that other important pathways regulating cytoskeletal structure and organization are also activated by *ras*. Rac1 regulates membrane ruffling in response to growth factor stimulation (Ridley *et al*., 1992), while RhoA functions in the regulation of actin stress fiber and focal adhesion assembly in growth factor-stimulated cells (Ridley and Hall, 1992). It appears that activation of both the MAPK and Rac/Rho pathways is required for full transformation by *ras* (Prendergast *et al*., 1995; Khosravi-Far *et al*., 1995; Qiu *et al*., 1995a,b). In light of this recent evidence, we are currently investigating activation of the Rac/Rho pathway in response to our integral membrane versions of *ras*.

Materials and methods

Construction of fusion proteins

Full-length Harvey p21*ras*^{WT} and p21*ras*^{61L} cloned into pcDNAI at the HindIII-EcoRI sites were generously provided by J Buss. Restriction sites in the coding sequences of *ras*^{WT} and *ras*^{61L} used to make these clones are as follows: BsaHI at nt 28 and FspI at nt 215 were used in generation of clones with E1 or E1(QI) transmembrane anchors; AflIII at nt 470 was utilized to generate mutations in the C-terminus. Note that the mutation resulting in the Gln→Leu at codon 61 occurs at nt 182, and consists of a A→T transversion. All constructs containing N-terminal transmembrane domains and/or C-terminal mutations described in this paper were constructed using the following strategy. Pairs of complementary oligonucleotides were designed and synthesized such that, when annealed, overhangs for restriction sites were formed. More details about the making of the various fusion constructs are available upon request. All synthetic oligonucleotides were gel purified as previously described (Xu *et al*., 1993), and all DNA sequences derived from oligonucleotides were confirmed by dideoxy nucleotide sequencing before use.

Focus assays

NIH3T3 cells were maintained as previously described (Hart *et al*., 1994). Cells were split at a density of 2×10^5 cells per 60 mm plate and transfected the following day using the calcium phosphate precipitation protocol (Chen and Okayama, 1987). Cells from each 60 mm plate were split 1:12 2 days later, and scored for foci 12–14 days later. These assays were repeated at least 2× for each construct.

Indirect immunofluorescence

NIH3T3 cells were split 1×10^5 onto 60 mm plates containing glass coverslips and transfected the following day with 10 μg of plasmid DNA, as described above. Two days after transfection, the cells were fixed with 3% paraformaldehyde/PBS and permeabilized with 0.5% Triton X-100/PBS. The intracellular location of *ras* fusion proteins was detected with rat monoclonal antiserum Ab-2 (Y13-238) directed against v-H-*ras* (Oncogene Science) and fluorescein-conjugated goat α-rat secondary antibody (Boehringer Mannheim).

For double-labeling experiments, cells were fixed and permeabilized as described above. *ras* fusion proteins were detected as described above, then the coverslips were treated with monoclonal Ab 10E6, which detects the *cis*-Golgi of cells (kindly provided by WJ Brown and V Malhotra) and rhodamine-conjugated goat α-mouse secondary antibody (Boehringer Mannheim).

Immunoprecipitation

NIH3T3 cells were split 2×10^5 onto 60 mm plates and 2 days after transfection, monolayers were washed 2× with Tris-saline, incubated 15 min with DME minus Cys and Met, and then labeled for 2 h with 100 μCi each of [^{35}S]-Cys and [^{35}S]-Met. Cells were lysed in RIPA (1% Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl pH 7.5, 0.1% SDS, 1% DOC, 10 μg/ml aprotinin), precleared with Protein A-Sepharose, and incubated with Ab-2 rat monoclonal antibody. Immunoprecipitates were collected with Protein A-Sepharose beads coated with rabbit α-rat IgG, washed 4× with RIPA, and resuspended in 2× sample buffer (50 mM Tris pH 6.8, 2% SDS, 20% 2-mercaptoethanol, 10% glycerol). Proteins were separated by 15% SDS–PAGE and detected by fluorography.

GTPase assays

NIH3T3 cells were transfected as described for immunoprecipitation. Two days after transfection, monolayers were lysed with lysis buffer (20 mM Tris-HCl pH 7.5, 0.125 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 10 μg/ml aprotinin) and precleared with Protein A-Sepharose. One third of the total lysate was then subjected to immunoprecipitation using Ab-2. Immune complexes were collected on Protein A-Sepharose beads coated with rabbit α-rat IgG, washed 2× with lysis buffer, 2× with RIPA, then incubated for 30 min on ice with 100 μl of 1.0×10^{-7} M α-[^{32}P]GTP in RIPA. Beads were then washed 3× with RIPA, 1× with lysis buffer, and incubated at 37°C for 1 h in 100 μl of lysis buffer. 2 μl of each sample was spotted onto PEI-cellulose plates (JT Baker, Phillipsburg, New Jersey) and chromatographed in 0.75 M KH_2PO_4, pH 3.4.

Abbreviations

E1, avian coronavirus E1 glycoprotein; ER, endoplasmic reticulum; IBV, infectious bronchitis virus.

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