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THE FARNESYL GROUP ACTIVATES RAS TOWARD GUANINE NUCLEOTIDE EXCHANGE CATALYZED BY THE SOS PROTEIN

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Abstract: The farnesyl group and a number of its analogs were enzymatically incorporated into H-Ras and K-Ras in vitro in order to study the prenyl group dependence of the hSOS1-catalyzed nucleotide exchange of Ras. Farnesylation of H-Ras and K-Ras in the absence of further processing was sufficient to facilitate the exchange although fully processed Ras showed a somewhat higher rate of exchange. H-Ras containing 15-carbon analogs of the farnesyl group were good substrates for hSOS1, but geranylated-H-Ras was not.

The Ras GTP-binding proteins play a pivotal role in a variety of signal transduction and differentiation processes.[1,2] Ras is also involved in the generation of a number of human cancers, and several oncogenic point mutations of Ras are known.[3,4] Ras is activated by the conversion of the GDP-bound inactive form to the GTP-bound active form in response to various extracellular signals.[5] Ras proteins are part of a pathway which connects the growth factor receptors to a variety of serine/tyrosine kinases, such as MAP-kinase.[6] The nucleotide bound and hence the activity of Ras is controlled by a set of proteins known as guanine nucleotide exchange factors (GEFs).[5] hSOS1 is the GEF responsible for the activation of human Ras proteins, and it catalyzes the exchange of GDP for GTP, following stimulation by epidermal growth factor[7] and insulin.[8]

Ras proteins are part of a group of proteins that are post-translationally prenylated.[9] In the case of Ras this modification involves the attachment of the farnesyl group to the protein through a thioether linkage to a cysteine located four residues from the C-terminus, followed by removal of three carboxy-terminal amino acids and methylation of the newly exposed α–carboxyl group of the farnesyl cysteine residue.[10] Additionally, H-Ras and N-Ras, but not K-Ras, undergo palmitoylation at one or more upstream cysteine residues.[11] Although necessary for the normal and oncogenic functions of many proteins, including Ras,[12] the specific properties imparted by these post-translational modifications have, to a large extent, remained unclear. Prenylation of proteins has been implicated in membrane binding[11,13] and in protein-protein recognition.[14-16] Furthermore, the relative contribution of each of the processing steps is unknown in most cases.

Previously it was shown that the activity of hSOS1 toward Ras was dependent on the post-translational processing of the latter protein. hSOS1 was active on the fully processed form of Ras but was inactive on the unprocessed form of Ras.[17] The present study was designed to determine which processing step (i.e., farnesylation, proteolysis, or methylation) is responsible for this activity and to what extent it is dependent on the structure of the farnesyl group. To this end we enzymatically incorporated the farnesyl group and also a number

of its analogs into recombinant H-Ras and K-Ras in vitro in order to study the effect of farnesylation on the hSOS1-catalyzed nucleotide exchange.

Experimental

Ras proteins. Unprocessed H-Ras,[16] unprocessed K-Ras,[18] fully processed K-Ras,[18] and hSOS1[17] were prepared as previously described. Synthesis of the analogs was previously described.[16] Enzymatic incorporation of the farnesyl group and the analogs was as previously described[16] with the exception that the incubation mixture was not passed over spin columns.

SOS assays. hSOS1 assays were performed by diluting stock solutions (4-7 μM) of fully processed Ras,[18] unprocessed Ras or in vitro modified Ras[16] to twice the indicated final concentrations with buffer A (20 mM Tris/HCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, pH 8.0) containing twice the final indicated amount of CHAPS (vol = 10 μL). The reaction was initiated by addition of 10 μL of buffer B (20 mM Tris/HCl, 20 mM $MgCl_2$, 100 mM NaCl, pH 7.5) containing 1 mg/mL BSA, 1 mM DTT, 1 μM [^{3}H]-GTP (S.A. 30 Ci/mmol) or 1 μM α-[^{32}P]-GTP (S.A. 20-30 Ci/mmol) (both from Dupont/NEN) and 70 nM hSOS1. The assays were incubated for 1 h at 30-32 °C and the reaction was terminated by transferring the assays to ice with the addition of 0.4 mL cold buffer B followed by vortexing. This mixture was then filtered over nitrocellulose (0.45 μm, Schleicher & Schuell) contained within a vacuum manifold (MINIFOLD I, Schleicher & Schuell). The original assay tubes were rinsed once with 0.4 mL buffer B, which was then passed over the nitrocellulose followed by additional washes (8 × 0.4 mL) with buffer B. The dried nitrocellulose was cut into pieces corresponding to individual assays and dissolved in 0.5 mL ethylene glycol dimethyl ether, and the radioactivity bound was determined by liquid scintillation counting. The values reported are the hSOS1 catalyzed rate with the uncatalyzed rate subtracted (i.e., identical assay conditions but without the addition of hSOS1). All assays and controls were done in duplicate.

Results and Discussion

To determine if the farnesyl group, in the absence of further processing, was sufficient to promote the hSOS1-dependent nucleotide exchange of Ras, recombinant H-Ras and K-Ras were enzymatically farnesylated in vitro and tested as a substrate for hSOS1 . To determine the dependence of the hSOS1 activity on the structure of the farnesyl group a number of analogs were also enzymatically incorporated into H-Ras and tested with hSOS1 (Figure 1).

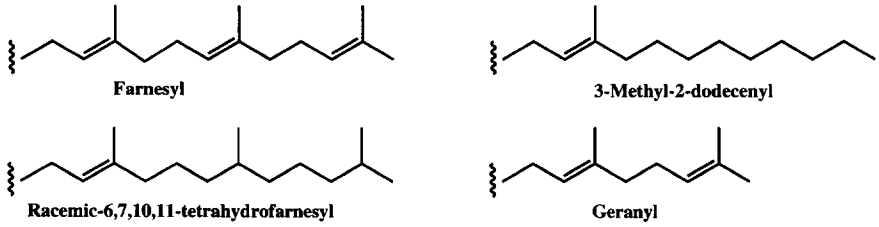

Figure 1. Structures of the farnesyl group and the analogs used.

It was found that farnesylation alone in the absence of further processing (proteolysis, methylation, palmitoylation) was sufficient to promote the hSOS1-catalyzed nucleotide exchange of H-Ras, although not to the same degree as fully processed Ras (Figure 2a). H-Ras modified by the tetrahydrofarnesyl or the methyldodecenyl group gave similar results to H-ras modified by the farnesyl group whereas H-Ras modified by the geranyl group and unprocessed H-Ras were inactive toward the hSOS1-catalyzed exchange (Figure 2b).

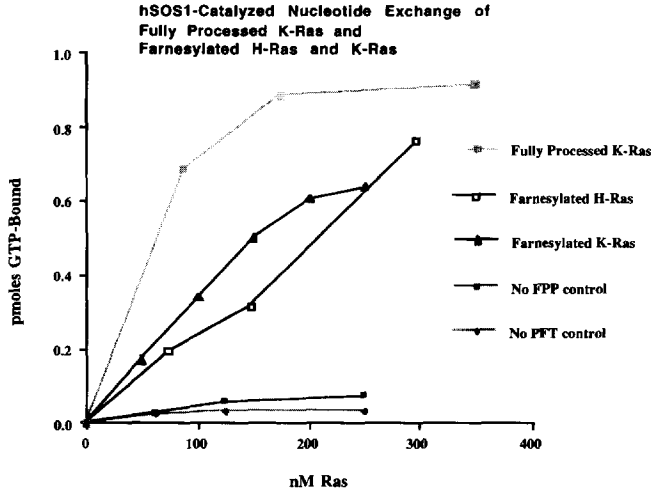

Figure 2a. hSOS1-catalyzed nucleotide exchange of fully processed K-Ras and farnesylated H-Ras (reactions mixture contained 0.3% CHAPS). Controls were carried out with nonprenylated H-Ras. This was prepared either by treating H-Ras with protein farnesyltransferase (PFT) in the absence of farnesyl pyrophosphate (FPP) (no FPP control) or by treating H-Ras treated with FPP in the absence of PFT (no PFT control).

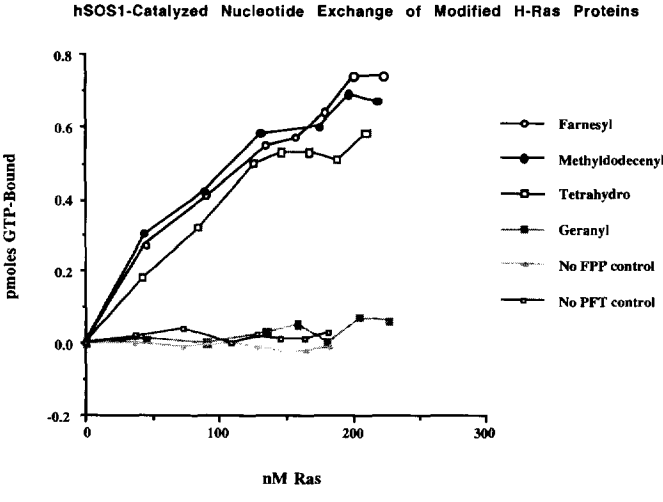

Figure 2b. Action of hSOS1 on farnesylated, analog-containing, or unprocessed H-Ras (reaction mixtures contained 0.03% CHAPS).

For maximal activity it was necessary to include detergent in the assays (data not shown). In order to compare the analogs while insuring that the activity was not dependent on partitioning of the protein into micelles, facilitated by the attached lipid, the lowest concentration of detergent giving maximal activity was used. It was necessary to use a higher concentration of detergent in experiments where fully processed protein was used, in order to maintain solubility of that protein. The activity was unchanged over a several fold variation in the detergent concentration and was inhibited at high concentrations of detergent at or near the CMC[19] (Figure 3). Data is shown for the farnesylated protein but fully processed material showed a similar detergent profile while the geranylated and the unprocessed Ras displayed a lack of activity at all detergent concentrations.

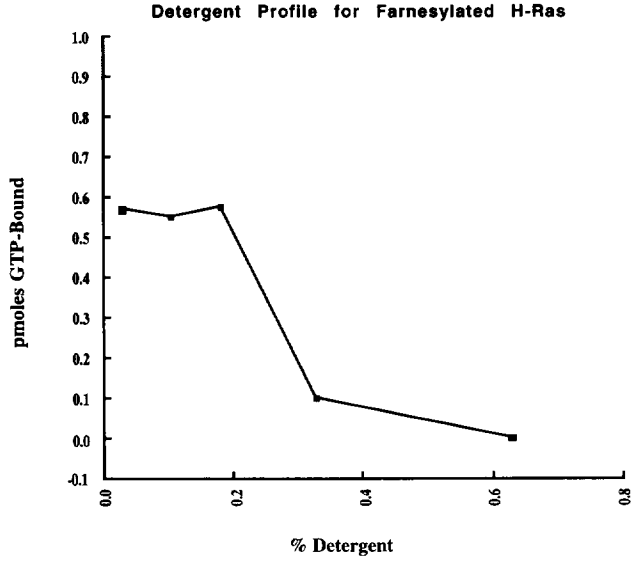

Figure 3. Detergent profile (CHAPS) of hSOS1-catalyzed nucleotide exchange. Farnesylated H-Ras was used at a concentration of 220 nM.

The results indicate that the hSOS1-catalyzed nucleotide exchange of Ras is dependent on the presence of the prenyl group and that the subsequent processing steps, while enhancing the rate, are of minor importance. It also appears that there is no strict dependence on the structure of the prenyl group for efficient exchange but that the ten carbon geranyl group is not sufficient to promote the exchange. Previously it was shown that hSOS1 enhances the rate of nucleotide exchange of fully processed H- or K-ras (i.e., farnesylated, proteolyzed and methylated) but not of the unprocessed forms of these proteins.[17] Here we demonstrate that it is primarily farnesylation that accounts for this activity. Furthermore, the earlier results are strengthened by the present studies because here we show that biologically inactive bacterially-produced H-Ras can be converted into an active form, with regard to hSOS1 activity, solely by farnesylation. The previous study used protein which could have been inactive for other reasons. These results are also consistent with our earlier results where it was found that

farnesylation of H-Ras alone, in the absence of further processing, was sufficient to cause the Ras-dependent activation of MAP-kinase in a soluble membrane-free system derived from *Xenopus laevis* oocytes.[16] In that system it was found that farnesylated H-Ras was about as active as the fully processed protein and that all of the analogs could support the activation although several, including geranyl were much poorer than farnesyl modified protein.

The results from the present study are consistent with a direct interaction of hSOS1 with the prenyl group of Ras. Alternatively, lipidation of the C-terminus of Ras may induce a structural change in the protein that is important for interaction with hSOS1.

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