whereas the recognition of c-Fos (a protein of the same family as Fra-1), MS2-polymerase (the protein in fusion) and β-galactosidase (a nonspecific protein that allows to control for nonspecific anti-Fra-1 reactivity as well as its reactivity to contaminating bacterial proteins) decreases. These results are confirmed by the Western blots shown in Figure 2, where anti-Fra-1 antiserum recognition of Fra-1 protein is significantly higher after immunopurification, with c-Fos recognition practically disappearing. Western blot assays also show that recognition of the MS2-polymerase protein levels decrease upon purification (data not shown). Figure 1B shows the results of purification of the anti-c-Fos antiserum by adsorption to extracts from bacteria producing MS2-polymerase-Fra-1. These results also show reproducible enrichment of the purified antibody response to c-Fos. Therefore, two specific antisera against two closely related proteins (c-Fos and Fra-1) were generated using this combined technique. These antisera are being used in our laboratory to investigate the role of c-Fos and Fos-related proteins in polyomavirus-induced malignant transformation (M.L.S. Oliveira and M.C.S. Armelin, unpublished).

REFERENCES


Quenching of Endogenous Peroxidase in Western Blot

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Enhanced chemiluminescence provides a simple, sensitive, nonradioactive method for the detection of antibody binding to Western blots. Sensitive chemiluminescent substrates have been developed for use with secondary antibodies conjugated with both alkaline phosphatase and horseradish peroxidase (HRP). Similar secondary antibodies have been utilized extensively for applications in immunohistochemistry. In histochemical preparations, endogenous enzymatic activities often require pre-quenching or inhibition. In the case of alkaline phosphatase, levamisol has been used to inhibit most endogenous activities. Similarly, endogenous HRP activities are routinely quenched in immunohistochemical preparations with pre-incubation with hydrogen peroxide (H₂O₂). Similar quenching or blocking steps have not been routinely recommended for enzymatic detection in Western blots.
Nevertheless, we have recently found that endogenous peroxidase activity can account for artifactual bands on Western blots. Proteins from subfractions of rabbit gastric mucosa were separated on Western blots and probed with antibodies using goat anti-mouse secondary antibodies conjugated with HRP (Promega, Madison, WI, USA). Labeling was detected using SuperSignal™ Substrate (Pierce, Rockford, IL, USA). An anomalous 30-kDa protein band was observed in Western blots of gastric proteins using a number of monoclonal antibodies, with the highest concentrations observed in membranes spun at 10,000×g. Since the distribution paralleled the pattern for mitochondrial membranes, we hypothesized that the artifact band might be peroxidase activity that had survived the separation methods of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting. Both the 10,000×g-spun membranes and recombinant rabbit Rab25 protein (1) were resolved on SDS-PAGE gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Immediately after transfer, blots were incubated in either water or 3% H₂O₂ for 15 min. Following the incubation, the blots were washed in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) and then blocked in 5% nonfat dry milk in TBS. Replicate blots were then incubated with (i) monoclonal anti-Rab25 antibodies followed by anti-mouse IgG secondary antibody conjugated with HRP, (ii) anti-mouse IgG secondary antibody conjugated with HRP or (iii) no primary or secondary antibodies. All blots received the same development according to the manufacturer. Figure 1 demonstrates that labeling of the recombinant Rab25 protein is only observed when blots have been incubated with both primary and secondary antibodies. The labeling of the recombinant by monoclonal antibodies was not affected by pre-incubation of the blot with H₂O₂. In contrast, a 30-kDa band was observed in gastric membranes spun at 10,000×g under all three conditions, verifying that the band represented endogenous peroxidase activity. However, all labeling of the 30-kDa protein was abolished when blots were pre-incubated with H₂O₂. This artifact is not specific to the Pierce chemiluminescence substrate, but was also apparent with the NEN Rennaisancethm substrate (NEN Life Science Products, Boston, MA, USA).

These results suggest that peroxidase activities can survive the process of sample preparation and resolution in SDS-PAGE and electrotransfer. In this case, the large number of mitochondria present in gastric parietal cells probably accounts for the peroxidase concentration in membranes spun at 10,000×g. Nevertheless, one can predict that peroxidase activities may present artifactual banding in Western blots in tissues with high mitochondrial numbers (e.g., liver or neutrophils), especially when cell fractionation is performed. Given the higher sensitivity of enhanced chemiluminescence detection, a small amount of peroxidase may confuse results centering on the detection of rare proteins. Fortunately, as in immunohistochemistry, these bands can be eliminated with pre-incubation of the blot with 3% H₂O₂ before primary antibody incubation.

Figure 1. Quenching of endogenous peroxidase in Western blots. 50 μg of gastric microsomes (G) spun at 10,000×g and 1 μg of recombinant Rab25 (R) were resolved on 15% SDS-PAGE gels and transferred to Immobilon-P. Following transfer, blots were incubated either with (A) water or (B) 3% H₂O₂ for 15 min. Replicate lanes were then processed for incubations with anti-Rab25 antibodies followed by HRP-conjugated anti-mouse IgG, HRP-conjugated secondary only, or with either primary or secondary antibodies. All blots were incubated with SuperSignal substrate. The positions of the molecular mass standards are indicated at the left.

REFERENCES


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